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Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins

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ABSTRACT **The majority of severe visual loss in the United States results from complications associated with retinal neovascularization in patients with ischemic ocular diseases such as diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity. Intraocular expression of the angiogenic protein vascular endothelial growth factor (VEGF) is closely correlated with neovascularization in these human disorders and with ischemia-induced retinal neovascularization in mice. In this study, we evaluated whether *in vivo* inhibition of VEGF action could suppress retinal neovascularization in a murine model of ischemic retinopathy. VEGF-neutralizing chimeric proteins were constructed by joining the extracellular domain of either human (Flt) or mouse (Flk) high-affinity VEGF receptors with IgG. Control chimeric proteins that did not bind VEGF were also used. VEGF-receptor chimeric proteins eliminated *in vitro* retinal endothelial cell growth stimulation by either VEGF ($P < 0.006$) or hypoxic conditioned medium ($P < 0.005$) without affecting growth under nonstimulated conditions. Control proteins had no effect. To assess *in vivo* response, animals with bilateral retinal ischemia received intravitreal injections of VEGF antagonist in one eye and control protein in the contralateral eye. Retinal neovascularization was quantitated histologically by a masked protocol. Retinal neovascularization in the eye injected with human Flt or murine Flk chimeric protein was reduced in 100% (25/25; $P < 0.0001$) and 95% (21/22; $P < 0.0001$) of animals, respectively, compared to the control treated eye. This response was evident after only a single intravitreal injection and was dose dependent with suppression of neovascularization noted after total delivery of 200 ng of protein ($P < 0.002$). Reduction of histologically evident neovascular nuclei per 6-μm section averaged 47% ± 4% ($P < 0.001$) and 37% ± 2% ($P < 0.001$) for Flt and Flk chimeric proteins with maximal inhibitory effects of 77% and 66%, respectively. No retinal toxicity was observed by light microscopy. These data demonstrate VEGF's causal role in retinal angiogenesis and prove the potential of VEGF inhibition as a specific therapy for ischemic retinal disease.**

Complications resulting from uncontrolled retinal angiogenesis account for most of the severe and irreversible visual loss associated with ischemic retinal disorders. These disorders include retinopathy of prematurity, diabetic retinopathy, retinal vein occlusion, and others, together accounting for the majority of new-onset legal blindness in the United States each year (1). For nearly one-half century, the retinal ischemia universally present in these conditions has been thought to promote the elaboration of angiogenic factors, ultimately resulting in neovascularization (2, 3). Recently, the molecule that has been variously referred to as vascular endothelial growth factor (4), vasopermeability factor (5), and vasculotropin (6) has been shown to possess many properties which suggest that it may mediate the majority of intraocular neovascularization associated with ischemic retinal disorders.

Vascular endothelial growth factor (VEGF) was originally described in highly vascularized tumors where its expression is increased by hypoxia (5, 7–9). VEGF is an endothelial mitogen (10), angiogenic protein (8–10), and potent vasopermeability factor (11, 12) that mediates its effects through the endothelial cell-specific, high-affinity, cell-surface transmembrane receptors *fms*-like tyrosine kinase (Flt) and fetal liver kinase 1 (Flk-1) (10, 13–15). Unlike molecules such as basic fibroblast growth factor, VEGF possesses a signal sequence and is secreted from intact cells (16, 17). In ocular tissues, studies have demonstrated that VEGF production is increased by hypoxia in retinal pigment epithelial cells, retinal endothelial cells, retinal pericytes (15, 18, 19), Müller cells (20), and both mouse and primate eyes with ischemia-induced retinal (20) and iris (21) neovascularization, respectively. Retinal endothelial cells possess numerous high-affinity VEGF receptors (13, 15). Recent clinical studies have demonstrated a close correlation between active ocular neovascularization and elevated intraocular VEGF concentrations in patients with diabetes mellitus, central retinal vein occlusion, retinopathy of prematurity, and rubeosis iridis (22, 23). However, a requirement for VEGF in the retinal neovascular response has not been proven.

Here we report that VEGF-neutralizing chimeric proteins, constructed by joining the extracellular domain of high-affinity VEGF receptors with the heavy chain of IgG, substantially reduce the development of retinal neovascularization when injected into the eyes of mice with ischemic retinal disease. This inhibition is specific to VEGF-receptor chimeric proteins, is dose dependent, and occurs in the absence of any histologically evident ocular toxicity or inflammation. These data suggest that VEGF serves a causal role in some forms of retinal angiogenesis. In addition, these studies support the potential of VEGF inhibition as an innovative therapy for certain ischemic retinal diseases, thereby circumventing the inherent retinal destruction produced by current laser photocoagulation and cryotherapy-treatment regimens (24, 25).

MATERIALS AND METHODS

Cell Cultures. Bovine retinal endothelial cells and retinal pericytes were isolated from fresh calf eyes by homogenization and a series of filtration steps as described (26, 27). Primary

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Abbreviations: VEGF, vascular endothelial growth factor; P7, etc., postnatal day 7, etc.

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endothelial cell cultures were grown in fibronectin (NYBen Reagents, New York Blood Center)-coated dishes (Costar) containing Dulbecco's modified Eagle's medium (DMEM) with 5.5 mM glucose, 10% plasma-derived horse serum (Wheaton, Scientific), 50 mg of heparin per liter and 50 units of endothelial cell growth factor per liter (Boehringer Mannheim). After the cells reached confluence, the medium was changed to include 5% fetal bovine serum (HyClone). Medium was changed every 3 days. Bovine retinal pericytes were cultured in DMEM/5.5 mM glucose with 20% fetal bovine serum.

Hypoxic Conditioned Medium. Confluent retinal pericyte monolayers were exposed for 24 hr to 2% O_2/5% CO_2/93% N_2 using a Lab-Line Instruments advanced computer controlled infrared water-jacketed CO_2 incubator with reduced oxygen control (model 480). All cells were maintained at 37°C and showed no morphologic changes by light microscopy, excluded trypan blue dye (>98%), and could subsequently be passaged normally. Cells incubated under normoxic conditions (95% air/5% CO_2) from the same batch and passage were used as controls. Medium was subsequently collected and filtered (Nalgene; 0.22 μm) prior to use.

Retinal Endothelial Cell Growth Assay. Bovine retinal endothelial cells were plated sparsely (≈2500 cells per well) in 24-well dishes (Costar) overnight in DMEM containing 10% calf serum (GIBCO). VEGF (25 ng/ml; Genentech) was added to the medium or the medium was replaced with conditioned medium the next day in the presence or absence of a 10-fold molar excess of chimeric protein as indicated in the text. After incubation at 37°C for 4 days, the cells were lysed in 0.1% SDS and DNA content was measured using Hoechst 33258 dye and a fluorometer (model TKO-100; Hoefer) (13).

VEGF-Receptor Chimeric Proteins and Controls. Two VEGF-receptor–IgG chimeric proteins were constructed using Pfu polymerase and PCR. One molecule contained the entire extracellular domain (758 residues) of the human high-affinity VEGF receptor Flt fused to the coding sequence for amino acids 216–443 of the human IgGγ1 heavy chain, while the other contained the entire extracellular domain of the murine VEGF receptor Flk-1 fused to a mouse IgG(γ2B) heavy chain (22, 28). These VEGF–IgG chimeric receptors have the same affinity for VEGF as full-length VEGF receptors (K_d = 20 pM) and bind free VEGF, preventing it from effectively interacting with native receptors on vascular endothelial cells. Control chimeric proteins were constructed in a similar manner using an entirely human CD4–IgG chimera as control for Flt–IgG and a monoclonal anti-gp120 antibody of the same IgG(γ2B) isotype as the murine chimeric protein for the Flk-1–IgG control. Endotoxin levels were <0.5 unit per mg of protein for each preparation.

Mouse Model of Ischemia-Induced Retinal Neovascularization. This animal model has been described (20, 29). Briefly, C57BL/6J mice were exposed to 75% O_2 from postnatal day 7 (P7) to P12 along with nursing mothers. At P12, the mice were returned to room air. Intraocular injections were performed at P12 and sometimes P14 as described below. At P17 the mice were sacrificed by cardiac perfusion of 4% paraformaldehyde in phosphate-buffered saline and the eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4°C before paraffin embedding.

Intraocular Injections. Mice were deeply anesthetized with tribromoethanol (Avertin) for all procedures. The lid fissure was opened using a no. 11 scalpel blade and the eye was proptosed. Intravitreal injections were performed by first entering the eye with an Ethicon TG140-8 suture needle at the posterior limbus. A 32-gauge Hamilton needle and syringe were used to deliver 0.5 μl of protein solution diluted in Alcon balanced salt solution through the existing entrance site. The eye was then repositioned and the lids were approximated over the cornea. Repeat injections were performed through a previously unmanipulated section of limbus 2 days later.

Neovascular Quantitation. Over 50 serial 6-μm paraffin-embedded axial sections were obtained starting at the optic nerve head. After staining with periodic acid/Schiff reagent and hematoxylin (20, 29), 10 intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. Eyes exhibiting retinal detachment or endophthalmitis were excluded from evaluation and accounted for <11% of those studied. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section by a fully masked protocol. The mean of all 10 counted sections yielded average neovascular cell nuclei per 6-μm section per eye. No vascular cell nuclei anterior to the internal limiting membrane are observed in normal, unmanipulated animals (29).

Statistical Analysis. All determinations were performed at least in triplicate and experiments were repeated a minimum of three times. Results are expressed as means ± SD for all experiments. Analysis of *in vitro* results was performed by nonpaired Student's t test. Analysis of *in vivo* results used the χ^2 test for categorical data and paired Student's t test or the Mann–Whitney rank sum test for quantitative data with unequal variance. A P value of <0.050 was considered statistically significant.

RESULTS

Chimeric VEGF-receptor protein effect on VEGF action *in vitro* was evaluated by using sparsely plated cultures of bovine retinal microvascular endothelial cells (13, 26), which undergo growth stimulation after addition of VEGF (13, 18). Stimulation of these cells with recombinant human VEGF (25 ng/ml) produced a characteristic 68% ± 1.6% increase in cellular DNA content after 4 days ($P < 0.001$) compared with unstimulated cells (Fig. 1*A*). This VEGF stimulatory capacity was entirely eliminated by simultaneous addition (5 μg/ml) of either human Flt–IgG ($P = 0.006$) or murine Flk-1–IgG chimeric protein ($P = 0.003$) but not by control proteins that were not directed against VEGF. Similarly, retinal endothelial cell growth was stimulated 128% ± 10% ($P = 0.002$) by conditioned medium from retinal pericytes cultured under hypoxic conditions known to induce VEGF expression (18). This growth stimulation was suppressed 89% ± 11% in the presence of human Flt–IgG at 5 μg/ml ($P < 0.001$) and 82% ± 12% with addition of murine Flk-1–IgG at 5 μg/ml ($P < 0.001$), while control proteins had no effect (Fig. 1*B*).

To evaluate whether VEGF-specific chimeric proteins could reduce retinal neovascularization *in vivo*, we used a highly reproducible murine model of ischemia-induced retinal neovascularization (29). C57BL/6J mice exposed to 75% O_2 from P7 to P12 experience extensive retinal capillary obliteration. When these mice are returned to room air on P12, the inner retina becomes relatively hypoxic, VEGF mRNA and protein levels are increased (20), and retinal neovascularization occurs in 100% of animals by P17 (29).

Intraocular injection of 200–500 ng of human Flt–IgG or 225–750 ng of murine Flk-1–IgG chimeric protein performed as the mice were returned to room air reduced histologically evident retinal neovascularization at P17 in 25 of 25 (100%; $P < 0.0001$) and 21 of 22 (95%; $P < 0.0001$) animals, respectively, compared with equivalent injection of control protein in the contralateral eye (Fig. 2). This effect was detectable at total chimera doses of 200 ng and above per eye, even when only a single intravitreal injection was performed. The magnitude of neovascular suppression was dose dependent (Fig. 3) with an inhibition of 47% ± 4% ($P < 0.001$) obtained when a maximally concentrated chimeric solution of Flt (250 ng) was delivered on both P12 and P14. Individual animals demonstrated up to 77% inhibition in retinal neovascularization. Dose-dependent neovascular inhibition was also observed with a single 225-ng injection of Flk-1–IgG (37% ± 2%; nine animals; $P < 0.001$) or a similar dual injection (46% ± 12%; three animals; $P = 0.050$; data not shown). Technical considerations in these neonatal mice precluded testing of more

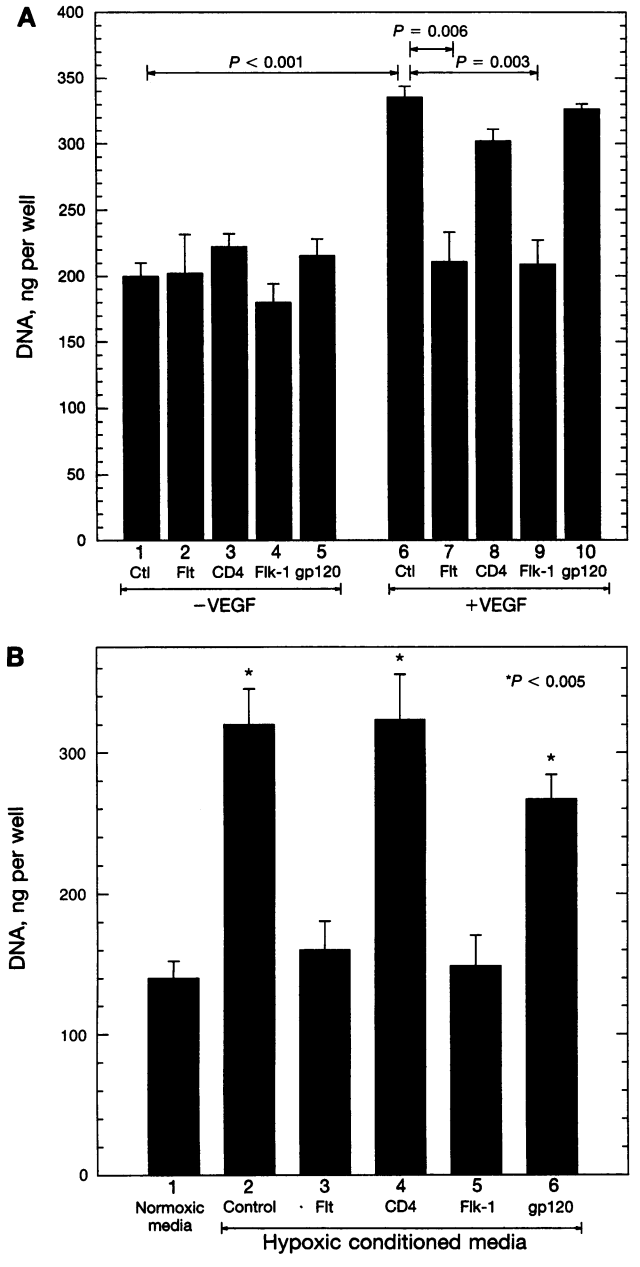

FIG. 1. Stimulation of retinal endothelial cell growth by VEGF (*A*) or hypoxic-conditioned medium (*B*) is inhibited by human and mouse VEGF receptor chimeric proteins. (*A*) Sparsely plated bovine retinal endothelial cells were exposed for 4 days to either no VEGF (bars 1–5) or recombinant human VEGF at 25 ng/ml (bars 6–10) in the presence of 10-fold molar excess (5 μg/ml) of concurrently administered human Flt–IgG chimera (bars 2 and 7; Flt), human CD4–IgG chimera (bars 3 and 8; CD4), murine Flk-1–IgG chimera (bars 4 and 9; Flk-1), or anti-gp120 monoclonal antibody of the same IgG(γ2B) isotype as the murine Flk-1–IgG chimeric protein (bars 5 and 10; gp120). DNA content was measured after cell lysis in 0.1% SDS (13). (*B*) Conditioned media were collected from retinal microvascular pericytes cultured under hypoxic conditions (2% O_2/5% CO_2/93% air) at 37°C for 24 hr (18, 27), filtered, and applied to sparsely plated bovine retinal endothelial cells for 4 days (bars 2–6) in the presence of human Flt–IgG chimera (bar 3; Flt), human CD4–IgG chimera (bar 4; CD4), murine Flk-1–IgG chimera (bar 5; Flk-1), or murine anti-gp120 monoclonal antibody (bar 6; gp120). Normoxic pericyte conditioned medium was used as a control in bar 1. Error bars indicate standard error of triplicate samples from a representative experiment. Experiments were repeated three times with similar results.

concentrated solutions or more frequent administration. Suppression of the neovascular response was evident by histologic

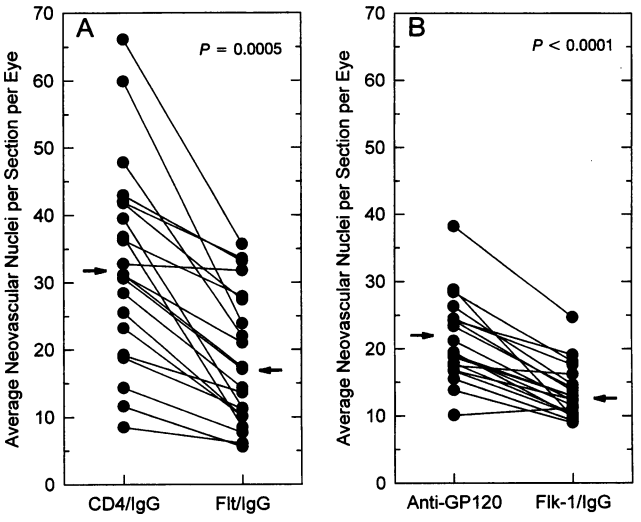

FIG. 2. Inhibition of VEGF suppresses neovascularization of ischemic mouse retina *in vivo*. To induce neovascularization, C57BL/6J mice with nursing mothers were exposed at P7 to 75% oxygen for 5 days and then returned to room air (29). Immediately upon return to room air, the left eye was injected intravitreally with 100–250 ng of human Flt–IgG chimera (*A*) or 225–375 ng of murine Flk-1–IgG chimera (*B*), while the right eye was injected with equivalent doses of either human CD4–IgG chimera or murine anti-gp120 monoclonal antibody controls, respectively. Eyes were reinjected on P14 and enucleated on P17. Average neovascular cell nuclei per 6-μm histological section per eye were determined as described. Eyes from the same animal are connected by solid lines, and arrows mark the mean of each group.

examination of paraffin-embedded ocular cross-sections (Fig. 4). No retinal toxicity or inflammation was apparent by light microscopy.

DISCUSSION

Our initial data demonstrated that VEGF receptor chimeric proteins could prevent *in vitro* stimulation of retinal endothelial cell growth by both exogenous and hypoxia-induced

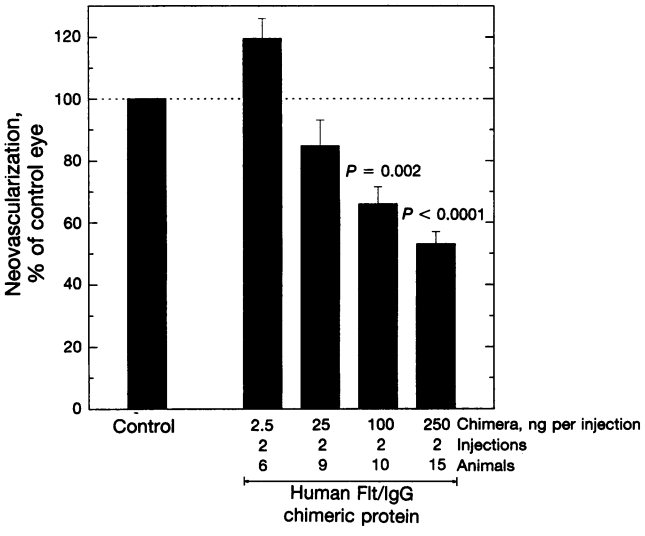

FIG. 3. Inhibition of retinal neovascularization is dose dependent on extent of VEGF inhibition. Retinal neovascularization determined from neovascular nuclei counts of chimeric treated eyes is expressed as percent of right eye control for each treatment regimen. Intravitreal injections of 0.5 μl each were performed on P12 and P14. Error bars indicate standard error for all animals in each group. Statistical differences compared with control eyes are indicated.

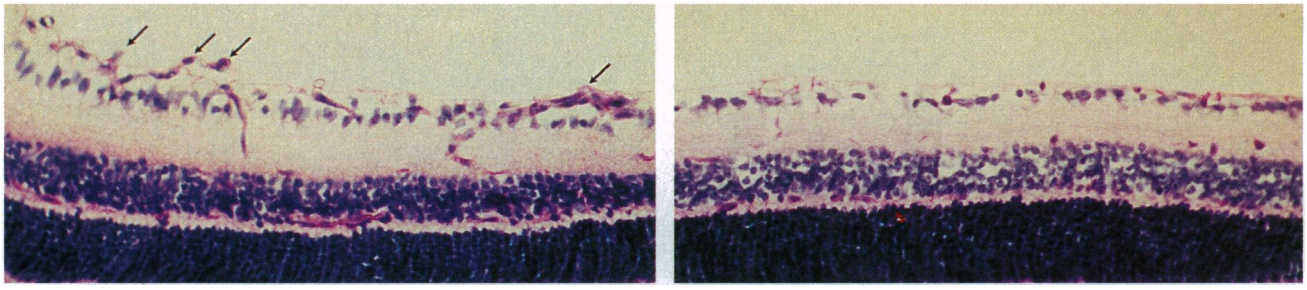

FIG. 4. Soluble VEGF receptor–IgG chimeric proteins reduce histologically evident ischemia-induced retinal neovascularization. Retinal ischemia was induced in C57BL/6J mice as described in Fig. 2. The right eye of each mouse was injected with 250 ng of human CD4–IgG control chimeric protein on P12 and P14 (*Left*). The left eye received intravitreal injections of 250 ng of human Flt–IgG chimera at the same times (*Right*). Paraffin-embedded, periodic acid/Schiff reagent, and hematoxylin-stained 6-μm serial sections were obtained as described. Typical findings from corresponding retinal locations from both eyes of the same mouse are shown and are representative of all animals studied. Vascular cell nuclei internal to the inner limiting membrane represent areas of retinal neovascularization and are indicated with arrows. No vascular cell nuclei anterior to the internal limiting membrane are observed in normal, unmanipulated animals (29). (×50.)

VEGF. Since VEGF expression had been closely linked to active retinal neovascularization in numerous human ischemic retinal disorders, this finding suggested that these molecules might be capable of suppressing retinal neovascularization *in vivo*. To demonstrate that inhibition of VEGF is sufficient to reduce retinal neovascularization *in vivo*, we have used a highly reproducible murine model of ischemia-induced retinal neovascularization (29). During hyperoxic exposure, these neonatal mice experience extensive retinal capillary obliteration. When they are returned to room air, the inner retina becomes relatively hypoxic, VEGF mRNA and protein levels are increased (20), and retinal neovascularization occurs in 100% of animals (29). Thus, this mouse model resembles retinopathy of prematurity, which occurs in prematurely born human infants in whom the retina is incompletely vascularized, presumably resulting in ischemia, VEGF induction, and retinal neovascularization (22, 25). In addition, the VEGF elevation and increase in vascular permeability observed in the mouse model resemble these same characteristic findings observed in proliferative diabetic retinopathy and other ischemic retinal disorders (22).

Our results demonstrate that soluble VEGF-binding chimeric proteins can reduce ischemia-induced retinal neovascularization *in vivo* without discernible short-term retinal toxicity. Thus, VEGF appears to be important for development of ischemia-induced retinal angiogenesis. Since VEGF levels increase in these disorders (20–22), it appears that VEGF could be one of the direct causative factors for clinically observed neovascularization in certain ischemic retinal diseases.

The finding that ≈50% of retinal neovascularization was not inhibited by anti-VEGF treatment could imply that other angiogenic substances account for the remaining stimulatory activity. However, the large chimeric proteins used in these studies would not be expected to diffuse into the retinal tissues, thus limiting their inhibitory effects to that portion of VEGF secreted into the vitreous fluid, which subsequently acts on the retinal capillaries on the inner retinal surface. VEGF, however, is secreted within the substance of the retina (20) and certain isoforms are freely diffusible (17). Thus, activity of intraretinal VEGF may not be inhibited by intravitreal injection of chimeric proteins and could account for the remaining angiogenic activity. In larger eyes, the potential for delivery of more concentrated solutions or more frequent administration of inhibitors might further reduce neovascular activity.

Current clinical treatment for active intraocular neovascularization involves destruction of peripheral retinal by either laser scatter photocoagulation (24, 30) or cryotherapy (25). The beneficial effect is presumably mediated by relatively improved perfusion resulting from the same blood flow to less remaining viable retina and by increased diffusion of oxygen from the choroid to the inner retina through thinned retinal scars (31). Although often effective, these procedures induce multiple side effects because of their inherently destructive nature, including decreased peripheral vision, poor night vision, and impaired color perception (24). In addition, the disease processes may progress despite timely and exhaustive therapy (24, 25). The findings presented here suggest that inhibitors of VEGF action may be used to prevent some types of intraocular neovascularization without the retinal destruction inherent in current therapeutic modalities.

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